AD_____

Award Number:

W81XWH-09-1-0450

TITLE:

Neuroendocrine Differentiation in Prostate Cancer: Role of Bone Morphogenetic
Protein-6 and Macrophages

PRINCIPAL INVESTIGATOR:
Isaac Yi Kim, MD, PhD
CONTRACTING ORGANIZATION:

UMDNJ - Robert Wood Johnson Medical School,
Piscataway, NJ 08854

REPORT DATE:
July 2010

TYPE OF REPORT:
Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
               Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

     x Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

1. REPORT DATE *(DD-MM-YYYY)* 01-07-2010	2. REPORT TYPE Annual	3. DATES COVERED *(From - To)* 15 JUN 2009 - 14 JUN 2010
4. TITLE AND SUBTITLE Neuroendocrine Differentiation in Prostate Cancer: Role of Bone Morphogenetic Protein		5a. CONTRACT NUMBER
		5b. GRANT NUMBER W81XWH-09-1-0450
		5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S) Isaac Yi Kim, MD, PhD		5d. PROJECT NUMBER
		5e. TASK NUMBER
		5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) UMDNJ - Robert Wood Johnson Medical School Piscataway, NJ 08854		8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSOR/MONITOR'S ACRONYM(S)
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
In the grant proposal we have hypothesized that tumor-derived bone morphogenetic protein-6 (BMP-6) induces tumor associated macrophages (TAMs) to express interleukin-6 (IL-6) via a crosstalk between the Smad-dependent and the p38 pathway; IL-6 in turn drives neuroendocrine (NE) differentiation of prostate cancer cells. To test this proposal, three specific aims were proposed: 1) To investigate the mechanism of NE differentiation induced by BMP-6 in vivo. 2) To investigate the mechanism of IL-6 induction by BMP-6 in macrophages. 3)To study the efficacy of dorsomorphin, a small molecule inhibitor of BMP signaling, on NE differentiation of prostate cancer in vivo. To date, aim 1 has been completed while aim 2 is currently progressing. When BMP-6 overexpressing prostate cancer cell line Tramp-BMP6 was injected subcutaneously into IL-6 knockout (KO) and conditional macrophage-null mice, neuroendocrine differentiation was no longer observed. Mechanistically, series of studies including shRNA knockdowns and immunoprecipitation assays have confirmed that Smad5 and GAT4 interact to induce IL-6 expression in macrophages.

15. SUBJECT TERMS
Prostate cancer, bone morphogenetic protein-6, macrophages, interleukin-6

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	15	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

2

INTRODUCTION

Prostate cancer is the most commonly diagnosed and the second leading cause of cancer deaths in the US (Jemal et al., 2009). Although surgery and radiation are quite effective for an organ-confined disease, the outlook for patients with a metastatic prostate cancer is still bleak due to the inevitable emergence of hormone refractory prostate cancer (HRPC). Since neuroendocrine (NE) cells are androgen receptor negative and frequently found in HRPC (Abrahamsson et al., 1998), understanding the biology of NE cells in prostate cancer may uncover new targets of intervention in patients with HRPC. In this regard, we have observed that the NE cells in human prostate cancer tissues were frequently positive for BMP-6 and co-localized with tumor associated macrophages (TAMs). In tissue culture, co-culture of macrophages and prostate cancer cells led to the expression of NE markers by prostate cancer cells in the presence of BMP-6. A survey of potential macrophage-derived mediators of NE differentiation demonstrated a dramatic induction of interleukin-6 (IL-6) by BMP-6. In investigating the mechanism of IL-6 induction by BMP-6 in macrophages, we have unexpectedly observed that both the canonical BMP-signaling pathway mediated by Smads and the non-canonical Smad-independent pathway mediated by p38 are required. Based on these results, the present grant proposal was funded to further dissect the mechanism as well as the biology of the tumor-host interaction loop composed of BMP-6, TAMs, and IL-6 in the context of NE cells in prostate cancer.

BODY

Based on the preliminary data, we have hypothesized that **tumor-derived BMP-6 induces TAMs to express IL-6 via a crosstalk between the Smad-dependent and the p38 pathway; IL-6 in turn drives NE differentiation of prostate cancer cells.** To test this hypothesis, 3 specific aims were proposed: 1) To investigate the mechanism of NE differentiation induced by BMP-6 in vivo; 2) To investigate the mechanism of IL-6 induction by BMP-6 in macrophages; 3) To study the efficacy of dorsomorphin, a small molecule inhibitor of BMP signaling, on NE

differentiation of prostate cancer in vivo. The past year was spent on completing aim 1 and part of aim 2.

To investigate the role of IL-6 and macrophages on NE differentiation of prostate cancer cells in vivo, Tramp C2 cells expressing BMP-6 under the control of a tetracycline promoter was established. This cell line, designated as Tramp-BMP6 was injected subcutaneously into 20 wild type B6 mice; Tramp C2 cells were originally derived from B6 mice. Two weeks later when tumors became palpable, mice were divided into 2 groups of 10 each. Then to the experimental group, BMP-6 was induced by supplying doxycycline in the drinking water. After an additional 2-week period, all animals were sacrificed and tumors were harvested. Confocal immunofluorescence microscopy for NSE and BM8 (a murine macrophage marker) demonstrated the co-localization of NE cells and macrophages (Fig 1, top 2 panels). Next, an identical experiment was carried out in IL-6 KO mice (B6 background). The results revealed the absence of NE differentiation in the presence of macrophages (BM8+) (Fig 1, middle 2 panels). To assess the role of macrophages in vivo, the conditional macrophage-null cd11b-DTR transgenic mice were utilized (Duffield et al., 2005). These mice express the human diphtheria toxin receptor (DTR) under the control of cd11b, a macrophage-specific promoter. Because the mouse equivalent of DTR binds diphtheria toxin (DT) inefficiently, macrophage-specific expression of human DTR permits the specific elimination of macrophage with DT. Forty cd11b-DTR mice (B6 background) were injected with Tramp C2-BMP6 cells subcutaneously. Then, the animals were divided into four groups of 10 each – doxycycline-/DT-, doxycycline+/DT-, doxycycline-/DT+, and doxycycline+/DT+. In preselected groups, macrophages were removed by injecting DT intraperitoneally every other day for 2 weeks. The results revealed the absence of NE differentiation and macrophages (Fig 1, bottom 2 panels).

Simultaneously, studies were carried out to determine the mechanism of IL-6 induction by BMP-6 in macrophages. The preliminary data have demonstrate that the classical Smad pathway is necessary for BMP-6-induced IL-6 expression in macrophages. To determine the R-Smad that transduces BMP-6 signal for IL-6 expression, RAW 264.7 cells were co-transfected

with each of the R-Smads (Smad 1, 5, and 8) in combination with the co-Smad (Smad 4) and the IL6-Luc plasmid. As shown in Fig 2, the combination of Smad 1/4 increased IL-6 promoter activity more than any other R-Smad/Co-Smad combination. Interestingly, luciferase activity was consistently lower following the transfection of Smad 8/4. In a reverse experiment, the lentivirus-based shRNA approach was again used (Fig 3). As expected, the knock-down of Smad 1 expression led to the abrogation of IL-6 induction by BMP-6 while that of Smad 8 led to the induction of IL-6 promoter activity. These results, in sum, demonstrate that Smad 1 is the R-Smad that signals for IL-6 expression when macrophages are stimulated with BMP-6.

Preliminary data also suggested that the non-Smad pathway via GATA4 is involved in IL-6 induction by BMP-6 in macrophages. To determine whether GATA4 is involved in BMP-6 signaling in macrophages, siRNA was used. When GATA4 was knocked down in RAW 264.7, BMP-6 no longer induced the expression of IL-6 (Fig 4). Consistent with this observation, transfection of dominant-negative GATA4 (GATA4DN) also blocked the BMP6-induced IL-6 expression. Next, ChIP assay using the GATA3 and 4 antibodies revealed that only GATA4 binds to the BMP-6-response element in IL-6 promoter (Fig 5). This interaction between GATA4 and the BMP6-response element was disrupted by the p38 inhibitor SB203580 (Fig 6), demonstrating that GATA4 signals down-stream of p38. Next, confocal immunofluorescence microscopy demonstrated a simultaneous nuclear translocation of GATA4 and Smad 1 following BMP-6 treatment (Fig 7).

Based on these observations, we hypothesized that GATA4 and Smad 1 may complex to activate the transcription of IL-6 in the context of BMP-6 in macrophages. To test this concept, RAW 264.7 cells were transfected with varying combinations of Smads and GATA4 along with IL6-Luc. The results showed that the over-expression of GATA4 augmented Smad 1/4's capacity to induce IL-6 promoter activity (Fig 8). However, GATA4 alone did not increase IL-6 promoter activity. To further characterize the interaction between Smad 1 and GATA4, myc-tagged Smad 1, 4, 5, and 8 along with flag-tagged GATA4 were expressed in RAW 264.7 cells. Following the transfection, immunoprecipitation against the flag-tagged GATA4 followed by

immunoblot for the myc-tagged Smads demonstrated that Smad 1 interacted with GATA4 (Fig 9). In addition, Smad 4 and 8 also complexed with GATA4. Because the observed binding among Smads and GATA4 may be an artifact of overexpression, we next wanted to assay for the interaction of endogenous molecules. However, antibodies that reliably distinguish each of the R-Smads do not exist. Thus, myc-tagged Smads were overexpressed in RAW 264.7 cells and immunoprecipitation against myc-epitope followed by immunoblot for the endogenous GATA4 was performed. Following treatment with BMP-6, higher levels of GATA4 were immunoprecipitated out in lysates obtained from cells overexpressing Smad 1 and 4 (Fig 10). Interestingly, binding of Smad 8 to endogenous GATA4 did not change with the addition of BMP-6. These results along with the observation that overexpression of Smad 1/4 (but not Smad 8) led to an induction of IL-6 expression by BMP-6 demonstrate that GATA4 and Smad 1/4 are part of the transcription machinery that drives the expression of IL-6 in response to BMP-6. To further investigate interaction between endogenous Smad 1 and GATA4, shRNA knockdown experiments were performed. When Smad1 was knocked down, immunoprecipitation for GATA4 followed by immunoblot against Smad 1 demonstrated no protein band (Fig 11, top panel). Conversely, when GATA4 was knocked down, immunoprecipitation for Smad 1 followed by immunoblot against GATA4 revealed no positive protein band (Fig 11, bottom panel).

KEY RESEARCH ACCOMPLISHMENTS

1. In vivo experiments using IL-6 KO and cd11b-DTR conditional macrophage-null mice have confirmed the critical role of IL-6 and macrophages in BMP-6-induced neuroendocrine differentiation of prostate cancer cells.

2. BMP-6-induced IL-6 expression requires Smad 5 and GATA4.

3. Smad 5 and GATA4 interact physically.

REPORTABLE OUTCOMES

1. The in vivo data reporting the role of IL-6 and macrophages in BMP-6-induced

neuroendocrine differentiation is currently being prepared for publication.

2. The mechanism of BMP-6-induced IL-6 expression in macrophages has been submitted for

publication. Minor revisions have been recommended. Revised manuscript will be submitted in

1-2 months.

CONCLUSION

During the first year of funding, we have demonstrated that BMP-6 induces neuroendocrine

differentiation of prostate cancer cells via IL-6 and macrophages. The second year of funding

will focus on further clarifying the mechanism of IL-6 induction by BMP-6 in macrophages.

Simultaneously, preclinical studies will be initiated using a small molecule inhibitor of BMP

signaling to determine whether neuroendocrine differentiation of prostate cancer cells in

reversible.

REFERENCES

Abrahamsson, P.A., Cockett, A.T., and di Sant'Agnese, P.A. (1998). Prognostic significance of neuroendocrine differentiation in clinically localized prostatic carcinoma. Prostate Suppl *8*, 37-42.

Duffield, J.S., Tipping, P.G., Kipari, T., Cailhier, J.F., Clay, S., Lang, R., Bonventre, J.V., and Hughes, J. (2005). Conditional ablation of macrophages halts progression of crescentic glomerulonephritis. Am J Pathol *167*, 1207-1219.

Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., and Thun, M.J. (2009). Cancer statistics, 2009. CA Cancer J Clin *59*, 225-249.

APPENDIX

Figures
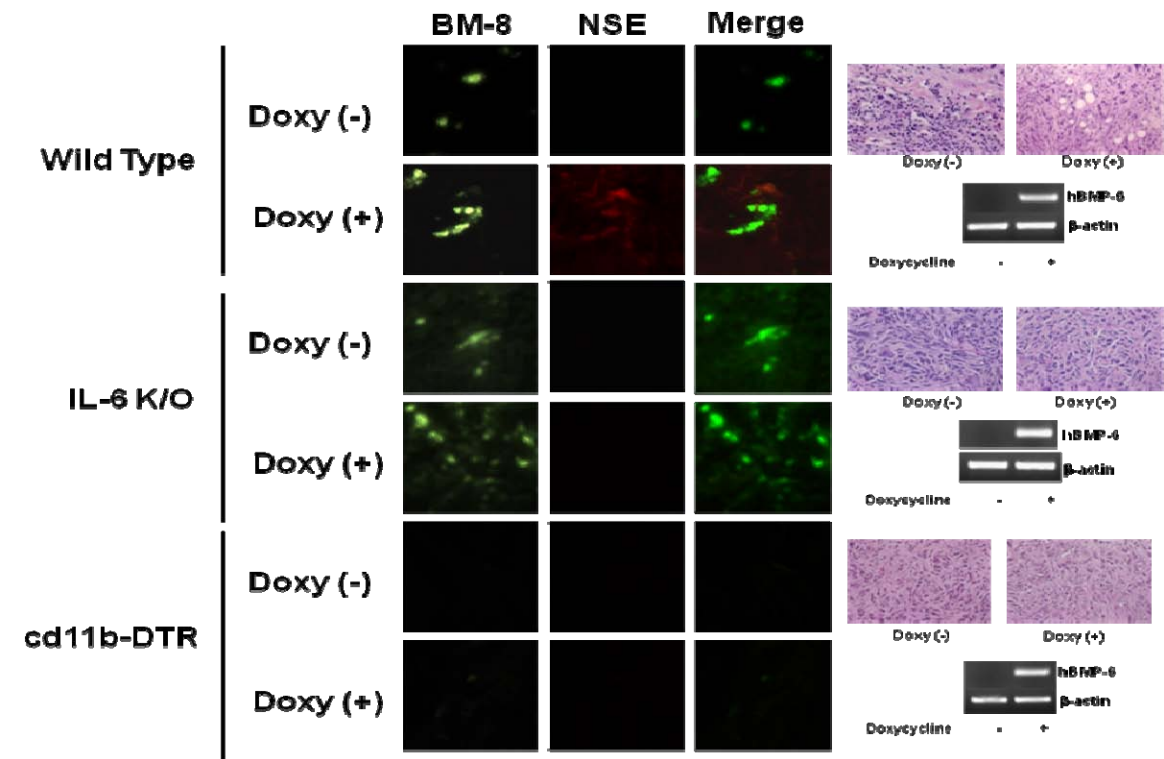
Fig 1. Tramp C2 cells expressing BMP-6 under the tetracycline-inducible promoter was injected subcutaneously into C57BL/6, IL-6 knock-out (IL-6 KO), and conditional macrophage-null (cd11b-DTR) mice. In cd11b-DTR mice, macrophages were selectively eliminated using the intraperitoneal injections of diphtheria toxin (DT). In the control C57BL/6 wild type mice, induction of BMP-6 expression with doxycycline demonstrated both macrophages (BM8+) and neuroendocrine differentiation (NSE+). In IL-6 KO mice, macrophages were present without neuroendocrine differentiation of prostate cancer cells. In cd11b-DTR mice, both macrophages and neuroendocrine differentiation were not observed. H&E staining demonstrated no obvious differences in tumor histology. The induction of BMP-6 was confirmed by RT-PCR.
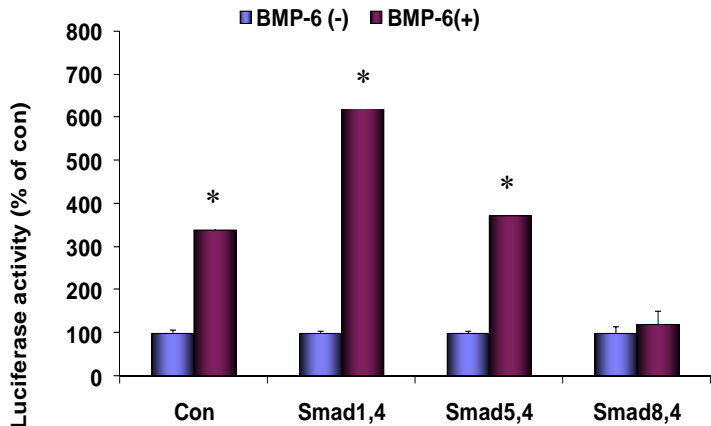

Fig 2. RAW 264.7 cells were cotransfected with each of the three R-Smads (Smad 1, 5, and 8) and the Co-Smad (Smad 4) along with IL6-Luc. When treated with BMP-6, cells expressing Smad 1/4

demonstrated the highest level of induction of IL-6 promoter activity. Interestingly, transfection with Smad 8/4 resulted in suppression of IL-6 promoter activity.
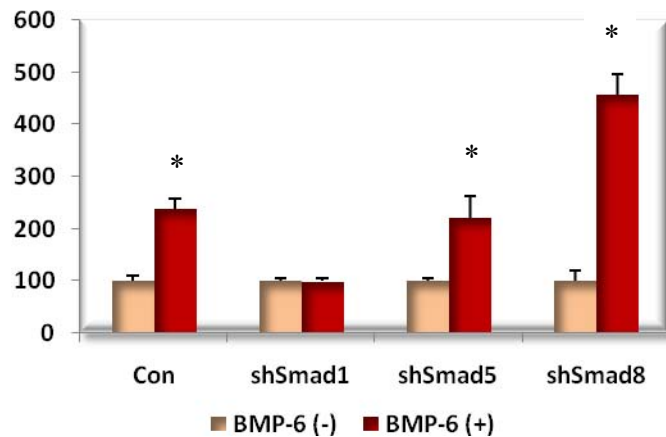

Fig 3. Lentiviruses containing shRNA sequences against Smad 1, 5, and 8 were infected into RAW 264.7 cells. Statistically significant knock-down of target gene expression was confirmed using RT-PCR. When transfected with IL6-Luc and treated with BMP-6, knock-down of Smad 1 blocked the induction of luciferase activity while that of Smad 8 increased luciferase activity.
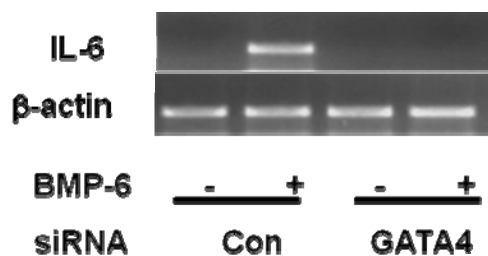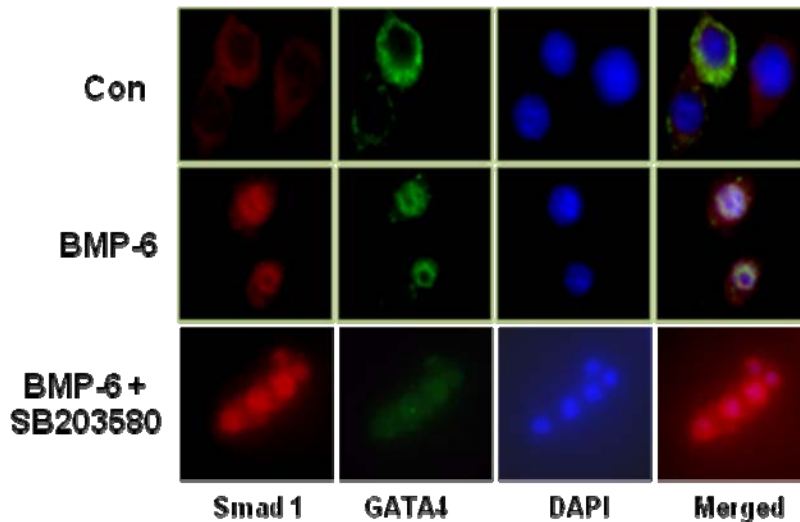

Fig 4. RAW 264.7 was transfected with GATA4 siRNA (siGATA4) and the effect on IL-6 expression was measured using RT-PCR. When treated with BMP-6, induction of IL-6 was no longer observed when GATA4 was knocked down.
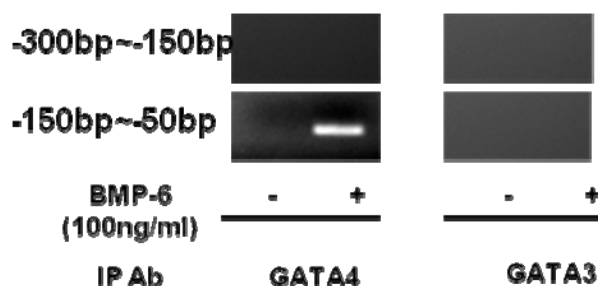

Fig 5. Chromatin immunoprecipitation was carried out to determine the interaction between GATA4 and IL-6 promoter. GATA3 was used as a control. Following treatment with BMP-6, the -50 to -150 bp region was amplified in samples immunoprecipitated with GATA4 but not GATA3 antibody.
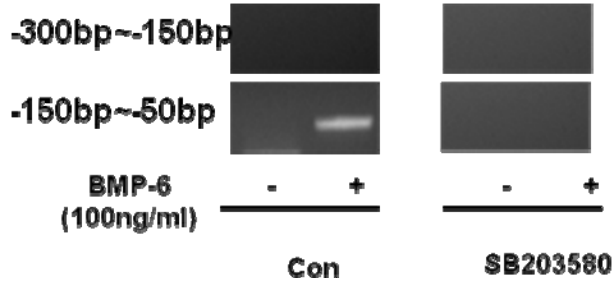
Fig 6. When RAW 264.7 cells were treated with SB203580 and BMP-6, ChIP assay using GATA4 antibody no longer amplified the -50 to -150 bp region. This observation demonstrates that GATA4 signals downstream of p38.


Fig 7. Confocal immunofluorescence microscopy was carried out using antibodies against Smad 1 and GATA 4. BMP-6 treatment induced nuclear translocation of Smad 1 and GATA4 simultaneously.
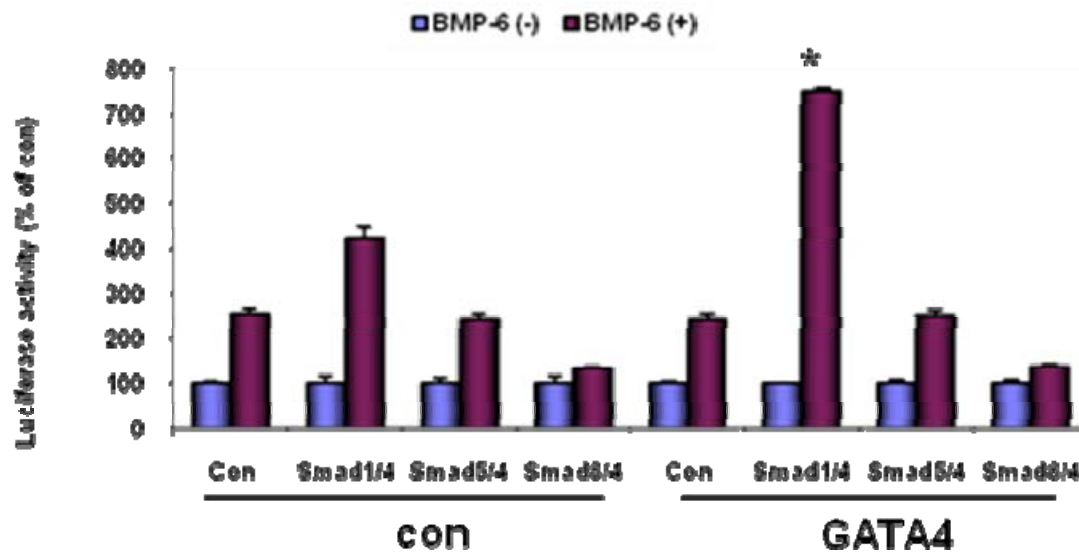
Fig 8. RAW 264.7 was transfected with indicated combination of Smad 1, 4, 5, and 8 and GATA4 along with IL6-Luc. BMP-6 induced a statistically significant level of IL-6 promoter activity when GATA4 was cotransfected with Smad 1/4. However, transfection of GATA4 alone did not induce IL-6 promoter activity (control bar in GATA4 group).
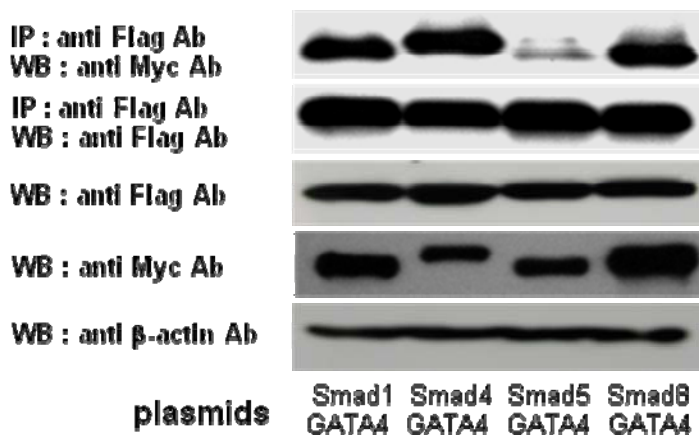
Fig 9. Myc-tagged Smads 1, 4, 5, and 8, along with flag-tagged GATA4 were expressed in RAW 264.7. Immunoprecipitation using anti-flag antibody followed by immunoblot analysis against myc epitope was performed. The results demonstrated that Smads 1, 4, and 8 but not 5 interacted with GATA4.
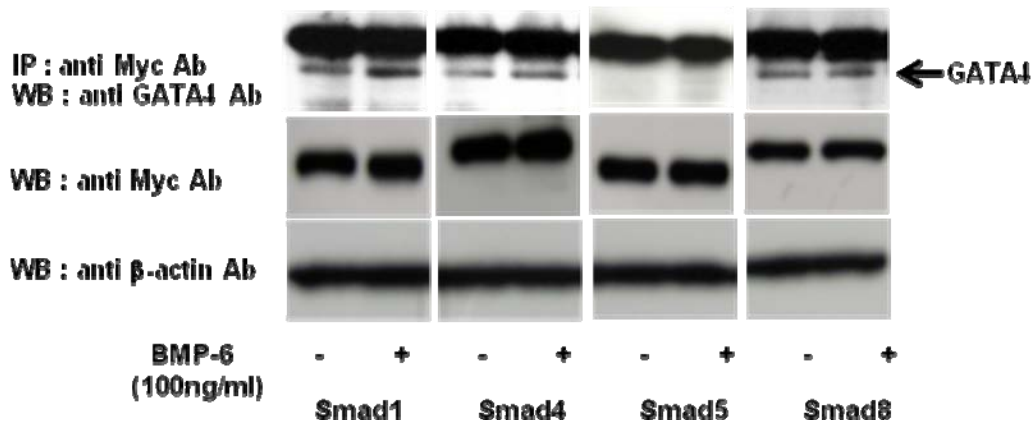
Fig 10. Myc-tagged Smads 1, 4, 5, and 8 were expressed in RAW 264.7. Immunoprecipitation using anti-myc antibody followed by immunoblot against endogenous GATA4 was performed. Following treatment with BMP-6, increased levels of GATA4 protein were immunoprecipitated out from cells transfected with Smad 1 or 4.
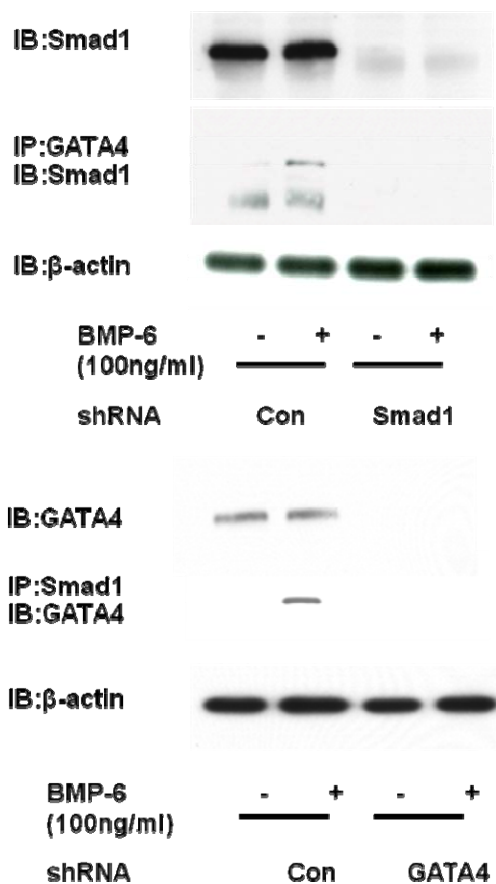
Fig 11. Interaction between endogenous Smad 1 and GATA4 was investigated using shRNA approach. When Smad 1 was knocked down and GATA4 was immunoprecipitated, immunoblot for Smad 1 revealed no protein band. Conversely, when GATA4 was knocked down and Smad 1 was immunoprecipiated, immunoblot for GATA4 demonstrated no protein band.